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RNA Interference Demonstrates a Role for *nautilus* in the Myogenic Conversion of Schneider Cells by *daughterless*

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Schneider SL2 cells activate the myogenic program in response to the ectopic expression of *daughterless* alone, as indicated by exit from the cell cycle, syncytia formation, and the presence of muscle myosin fibrils. Myogenic conversion can be potentiated by the coexpression of DMEF2 and *nautilus* with *daughterless.* In RT-PCR assays Schneider cells express two mesodermal markers, *nautilus* and DMEF2 mRNAs, as well as very low levels of *daughterless* mRNA but no *twist.* Full-length RT-PCR products for *nautilus* and DMEF2 encode immunoprecipitable proteins. We used RNA-i to demonstrate that both endogenous *nautilus* expression and DMEF2 expression are required for the myogenic conversion of Schneider cells by *daughterless.* Coexpression of *twist* blocks conversion by daughterless but *twist* dsRNA has no effect. Our results indicate that Schneider cells are of mesodermal origin and that myogenic conversion with ectopic expression of *daughterless* occurs by raising the levels of *daughterless* protein sufficiently to allow the formation of *nautilus*/*daughterless* heterodimers. The effectiveness of RNA-i is dependent upon protein half-life. Genes encoding proteins with relatively short half-lives (10 h), such as *nautilus* or HSF, are efficiently silenced, whereas more stable proteins, such as cytoplasmic actin or *β-galactosidase,* are less amenable to the application of RNA-i. These results support the conclusion that *nautilus* is a myogenic factor in *Drosophila* tissue culture cells with a functional role similar to that of vertebrate MyoD. This is discussed with regard to the *in vivo* functions of *nautilus.*

Key Words: **RNA interference; dsRNA; myogenesis; Schneider cells; *nautilus; daughterless;* DMEF2.**

INTRODUCTION

The MyoD-related proteins have been characterized not only in the vertebrates (see reviews: Arnold and Braun, 2000; Buckingham, 1997; Lassar *et al.*, 1994; Olson and Klein, 1998; Rudnicki and Jaenisch, 1995) but also in several invertebrate species, including *Drosophila* (Michelson *et al.*, 1990; Paterson *et al.*, 1991), *Caenorhabditis elegans* (Krause *et al.*, 1990), echinoderms (Venuti *et al.*, 1991), and ascidians (I. Araki *et al.*, 1994). Among invertebrates there is but a single member of the MyoD gene family. A hallmark in the activity of this family of transcription factors is the ability of these bHLH proteins to convert a variety of cell types to a myogenic fate (Davis *et al.*, 1987; Weintraub *et al.*, 1989). Transgenic and gene targeting studies in the mouse have clearly shown that the MyoD-related proteins are essential in order for muscle formation to occur (see reviews mentioned above). Classical genetic approaches as well as posttranscriptional gene silencing by the injection of double-stranded RNA, known as RNA interference (RNA-i), have established an essential role for the MyoD homologue, *hlh-1,* in *C. elegans* muscle formation and viability (Chen *et al.*, 1994; Fire *et al.*, 1998). However, deficiencies in *Drosophila* reported to remove *nautilus,* the MyoD homologue, are viable with essentially normal muscle (Keller *et al.*, 1998). In disagreement with this result, injection of dsRNA for *nautilus* into *Drosophila* embryos as well as overexpression of antisense *nautilus* RNA in the mesoderm using the Gal4/UAS system (Brand and Perrimon, 1993) demonstrated that *nautilus* is

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an essential gene for embryonic muscle formation and viability (Misquitta and Paterson, 1999).

Although there appears to be evolutionary conservation of MyoD function comparing MyoD activity of *C. elegans* and *Drosophila* (Zhang *et al.*, 1999), E proteins do not seem to play the same role in every instance (Krause *et al.*, 1997). The *C. elegans* MyoD homolog, *hlh-1*, can activate myogenesis in mouse fibroblasts independent of either vertebrate or *C. elegans* E proteins, whereas *nautilus* protein requires the E protein homologue, *daughterless*, for activation (Zhang *et al.*, 1999). This is due to the fact that *nautilus* protein does not heterodimerize efficiently with vertebrate E proteins (Shirakata *et al.*, 1993). In *C. elegans* embryonic muscle development, the E/*daughterless* homologue, *hlh-2*, is detectable in neuronal precursors but not in *hlh-1*-positive cells, suggesting that the *hlh-1* homodimer may be sufficient to drive myogenesis since there is no other E protein homologue in the worm (Krause *et al.*, 1997). To date there is no direct experimental evidence demonstrating that *nautilus* acts either as a homodimer or as a heterodimer to activate myogenic genes during *Drosophila* development. Even among the vertebrates, alternative strategies have evolved in the use of bHLH transcription factors, as evidenced by the differential utilization of the E protein, E47, in B cell differentiation versus myogenesis. The E47 protein activates B-cell-specific genes as a homodimer yet functions as a heterodimer with MyoD during vertebrate myogenesis (Shen and Kadesch, 1995; Sloan *et al.*, 1996; Weintraub *et al.*, 1994).

To determine if the myogenic program could be activated in *Drosophila* Schneider cells, cultures were transfected with various combinations of expression plasmids for *nautilus* (Michelson *et al.*, 1990; Paterson *et al.*, 1991), *daughterless* (Caudy *et al.*, 1988; Cronmiller *et al.*, 1988) and DMEF2 (Lilly *et al.*, 1994). The vertebrate homologues of these factors have been shown not only to play critical roles in myogenesis but also to synergize in the activation of muscle-specific genes (Black and Olson, 1998; Lassar *et al.*, 1991; Molkentin *et al.*, 1995). To test for myogenic conversion, transfected cultures were subsequently analyzed for muscle myosin heavy chain expression. Here we describe the myogenic conversion of Schneider cells by the ectopic expression of *daughterless* alone. Conversion is shown to be dependent upon the endogenous expression of both *nautilus* and DMEF2 but independent of *twist* by using RNA-i to inactivate the mRNAs for these genes. We further show that RNA-i can be used to target not only endogenous gene function but also transfected genes and reporter constructs. The *nautilus*/*daughterless*/DMEF2-dependent myogenic conversion of Schneider cells defines a novel system in which to study aspects of *Drosophila* myogenesis *in vitro* and provides supportive evidence that the *nautilus*/*daughterless* heterodimer, like its MyoD/E-protein vertebrate counterpart, plays an important role in *Drosophila* muscle formation. This is discussed in terms of the current genetic data and the results of RNA-i studies regarding the role of *nautilus* during *Drosophila* development.

MATERIALS AND METHODS

Cell lines. The *Drosophila* Schneider SL2 cell line, a clonal stock, was grown at 24°C in serum-free HyQ-CCM3 medium (HyClone No. SH30065.01) supplemented with gentamycin sulfate (50 μg/ml).

Plasmid DNA. The PCR-amplified coding regions for *nautilus*, DMEF2, *daughterless*, mouse PKR, and mouse P58 were cloned into the *Drosophila* expression plasmid pAct-PL (Thummel *et al.*, 1988) using the appropriate restriction sites on primers covering the initiator methionine and the translational stop codon for each protein. pUAS clones for *β-galactosidase* were activated with Gal4-VP16 cloned into pAct-PL. Nuclear GFP was cloned from the pShooter vector, pCMV/myc/nuc (Invitrogen No. V821-20) into pAct-PL. The cytoplasmic actin clone was from Ward Odenwald (NINDS, NIH).

Antibodies. Polyclonal rabbit antibodies to *Drosophila nautilus*, *daughterless*, DMEF2, and myosin heavy chain have been described previously (Misquitta and Paterson, 1999; Paterson *et al.*, 1991). Antibodies for HSF and Hsp26 were described previously (Marin *et al.*, 1993; Westwood *et al.*, 1991). Monoclonal antibody to *β-galactosidase* was from Gibco (No. 19929-017). Secondary antibodies were from Molecular Probes: Alexa Fluor 594 goat anti-mouse IgG (2 mg/ml) and Alexa Fluor 488 goat anti-rabbit (2 mg/ml). Tubulin antibody E7 was from the Developmental Studies Hybridoma Bank.

Transfection of SL2 cells with dsRNA and plasmid DNA. Cells were transfected using FuGENE 6 transfection reagent (Boehringer Mannheim No. 1814443). Newly confluent cultures were split 1:5 and grown to 70–80% confluent density on the day of transfection. Six to nine microliters of FuGENE 6 reagent was brought to 100 μl with serum-free medium in a sterile microfuge tube and incubated at room temperature for 5 min. In a second tube 2–3 μg of plasmid DNA, plasmid DNA plus 0.1 μg of dsRNA, or dsRNA alone was also adjusted to 100 μl with serum-free medium then diluted dropwise with the 100 μl of FuGENE 6 medium and incubated 20–30 min at room temperature prior to addition to the cells. Fresh medium was added to the cells prior to addition of the DNA: 3 ml for a 60-mm dish and 2 ml for chamber slides. The FuGENE 6 mixture was then added to the medium and the cells were fixed and stained 48 h later or as indicated.

Immunofluorescence. Cells were plated on glass chamber slides coated with poly-L-lysine (0.01% w/v in water). Cells were fixed for 20 min at room temperature in freshly prepared 4% paraformaldehyde in PBS. Cells were then washed with PBS and postfixed with 100% methanol at room temperature for 10 min, washed with PBS, and blocked for 30 min with PBS containing 3% BSA (w/v) and 0.1% Tween 20 (v/v). Cells were then incubated sequentially for 3 h at room temperature with primary antibody diluted in blocking buffer (1:500 for DMEF2, 1:1000 for MHC, 1:300 for *nautilus* and *daughterless*, 1:100 for *β-galactosidase*), washed with PBS, and then incubated for 1 h at room temperature with secondary antibody (1:200) in blocking buffer. After a brief wash with PBS the slides were mounted in Slow Fade (Molecular Probes) mounting medium. Samples were viewed with an Axioplan microscope equipped with the appropriate barrier filters and with a 63× phase 3 Plan-Apochromat objective. The confocal images were taken using a Bio-Rad MRC 1024 confocal microscope.

Preparation of dsRNA. DNA sequences to be utilized as dsRNA were amplified by PCR using *Pfu* (Stratagene) and primers with convenient restriction ends, cloned into BlueScript KS(+), and linearized with the appropriate restriction enzyme to give a 5′

overhang. Linearized plasmids were transcribed *in vitro* with T3 or T7 polymerases using the Ambion Megascript kits following the manufacturer's instructions. Transcripts were annealed as described previously (Misquitta and Paterson, 1999) and checked for size and nuclease resistance by incubation in 0.3 M sodium acetate with RNase A (10 μg/ml) and T1 (200 U/ml) at 37°C for 30 min.

RT-PCR analysis and DNA sequencing. Total Schneider cell RNA was prepared using the guanidine–phenol method (Chomczynski and Sacchi, 1987). RT-PCRs were carried out using the Access RT-PCR kit from Promega (Cat. No. A1250), as suggested by the manufacturer. For quantitative reactions the gel was stained with Cybergreen (Molecular Probes) and analyzed on the Fuji CCD camera (LAS1000). RT-PCR products representing the coding regions for *nautilus* and DMEF2 were amplified with an amino-terminal primer containing a T7 RNA polymerase binding site and a carboxy-terminal primer with 15 thymidine residues. The agarose gel-purified products were translated with [^{35}S]methionine in the Single Tube Protein System 3 from Novagen (Cat. No. 70192-3) or the TnT Quick T7 System from Promega (Cat. No. L1170) and analyzed on SDS–PAGE 10% gels. All RT-PCR products were sequenced on an ABI Prism 310 genetic analyzer using rhodamine dye-terminator cycle sequencing. The following primers were used for RNA analysis: *nautilus* (F-5′AAG GAT CCA TGG GCA AAA AGA AGA GCG TCA CCG3′/R-5′GCG AAT TCT TAC TCC AGG CTC TCG ATA TAC TCG3′), DMEF2 (F-5′AGG ATA GGA AAT CTG TTG CC3′/R-5′CAC GCC GTT CTT GTT CTC CTT3′), *daughterless* (F-5′GAA TTC GGG ACT GCA GCA GCA3′/R-5′CCG CCT TCG GAT TCA GGT TGC3′) and across the intron (F-5′GCT CAA CGT CAA CAC TCG CTG3′/R-5′GAA TCG AAA TAC TGA TCG ACGG3′), and *twist* (F-5′AGC AAG ATC CAG ACC CTG AA3′/R-5′AGG CAC TTC AGA TCT GCC TC3′). The following primers were used for expression of *nautilus* and DMEF2 from the full-size RT-PCR products as described (Cestari *et al.*, 1993): *nautilus* (F-5′TAA TAC GAC TCA CTA TG GGA GAC CAC CAT GAC CAA GTA TAA TAG TGG CAGC3′/R-5′TTT TTT TTT TTT TTT GCG GCC GCC TAA GTG CTG CAC TTC CGT TTG3′) and DMEF2 (F-5′TAA TAC GAC TCA CTA TAG GGA GAC CAC CAT GGG CCG CAA AAA AAT TCA AAT ATC ACG CAT CAC CG3′/R-5′TTT TTT TTT TTT TTT GCG GCC GCC TAT GTG CCC CAT CCG CCC GAT ATT CT3′).

RESULTS

daughterless Activation of the Myogenic Program in Schneider Cells

To assess whether Schneider cells can activate any myogenic markers, similar to the myogenic conversion of mouse 10T1/2 fibroblasts by MyoD (Davis *et al.*, 1987), various combinations of *nautilus*, *daughterless*, and DMEF2 were ectopically expressed in Schneider cells under the control of the *Drosophila* actin 5C promoter (Thummel *et al.*, 1988). Myogenic conversion was assayed using a rabbit polyclonal antibody to *Drosophila* skeletal muscle myosin heavy chain raised against a GST fusion to exon 17, an exon conserved in all *Drosophila* muscle myosins (Wassenberg *et al.*, 1987). As shown in Fig. 1, Schneider cells transfected with a *daughterless* expression plasmid can form multinucleated syncytia with two to four nuclei that also express skeletal muscle myosin in long fibrillar bundles with no sarcomere organization. Converted cells are sometimes elongated or extended with a flattened morphology. Furthermore, the nuclei in these syncytia are postmitotic since they are refractory to BrdU labeling (data not shown). The number of myogenic cells containing more than two nuclei represents less that 5% of the converted cell population. The majority of converted cells are binucleate, with single cells appearing less frequently. The reason for this is not clear. Ectopic expression of either *nautilus* or DMEF2 alone gave only weak, punctate myosin staining in single cells (Fig. 2). The coexpression of either *nautilus* or *nautilus* and DMEF2 along with *daughterless* did little to enhance the conversion phenotype observed with *daughterless* alone. However, the frequency of conversion was increased more than 10-fold with the coexpression of *nautilus* and *daughterless*, and the increase was maximal, around 40-fold, when both *nautilus* and DMEF2 were coexpressed with *daughterless* (Fig. 2 and Table 1). Similar synergy was observed with vertebrate MyoD and MEF2C in the myogenic conversion of mouse 10T1/2 fibroblasts (Molkentin *et al.*, 1995). Coexpression of *nautilus* and DMEF2 in Schneider cells gave no muscle phenotype (Table 1).

Endogenous Expression of Mesodermal Markers in Schneider Cells

One of the major limitations in the use of *Drosophila* cell lines in developmental studies stems from the lack of knowledge regarding the tissue derivation of these cells. We had previously shown that *nautilus* could convert mouse fibroblasts to a myogenic fate as long as the *Drosophila* E-protein homologue, *daughterless*, was present (Zhang *et al.*, 1999). This indicated that *nautilus* was likely functioning as a heterodimer in this context. Based upon this observation and the fact that *daughterless* alone triggered myogenic conversion, we speculated that Schneider cells might be expressing low levels of *nautilus* protein, even though antibody staining and Western blot analysis for *nautilus* were negative. To test for the presence of a functional *nautilus* transcript in Schneider cells, two types of experiments were performed. First, RT-PCR analysis was carried out with *nautilus* primers flanking the intron between the basic and the HLH domains (Michelson *et al.*, 1990) to check for the presence of spliced *nautilus* mRNA transcripts. Primers for *daughterless*, *twist*, and DMEF2 were also included in the initial tests to determine *daughterless* expression levels relative to *nautilus* and to check for the presence of other mesodermal markers. Where possible, primers were picked flanking an intron to produce RT-PCR products of 200–300 bp representing the spliced mRNA in question. All the RT-PCR products were sequenced to confirm the identity of the corresponding mRNA. As postulated, Schneider cells do express spliced *nautilus* mRNA (Fig. 3A, lanes 6 and 10 versus lane 13). Unexpectedly, however, mRNA for an additional mesodermal marker, DMEF2, was also detected (Fig. 3, lanes 5 and 9). No evidence was found for *twist* mRNA (data not

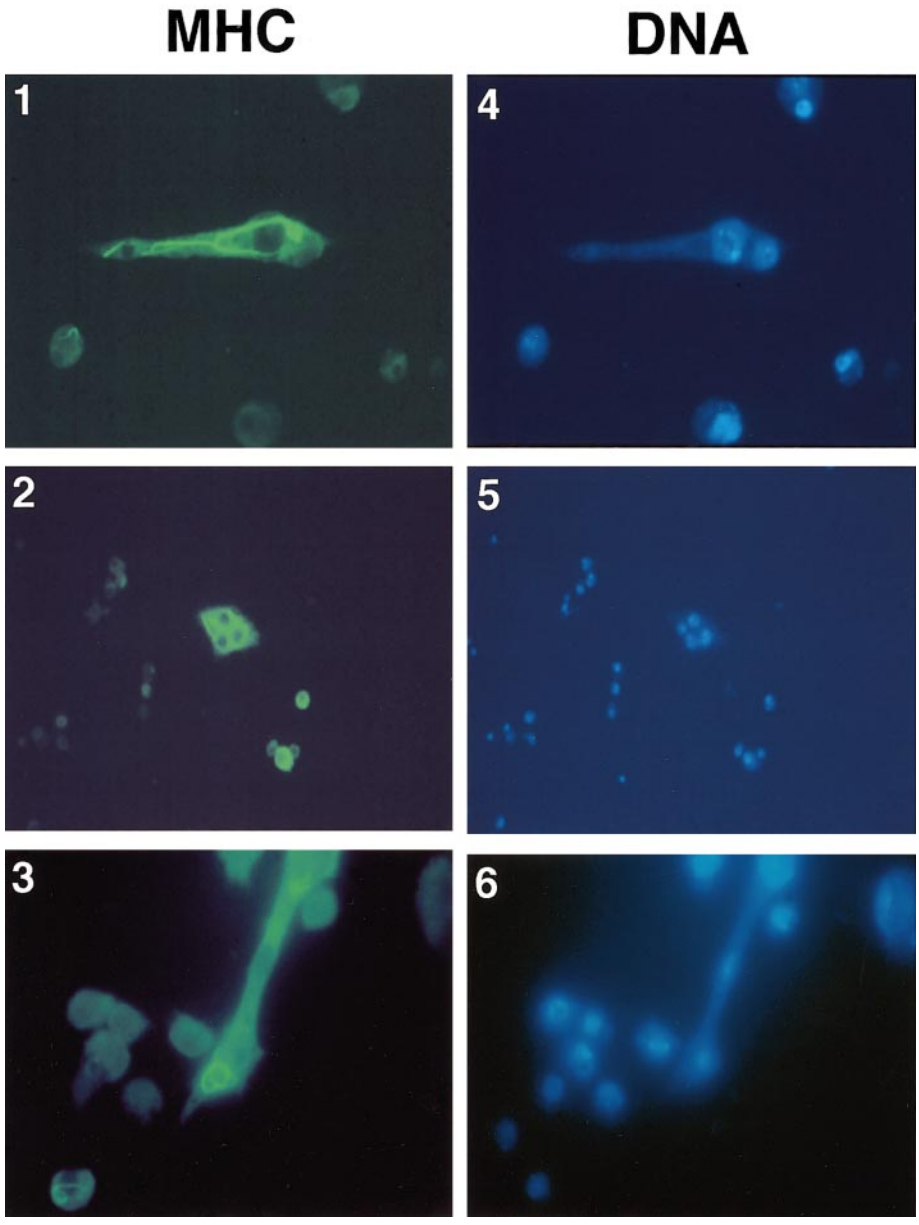

FIG. 1. Ectopic expression of *daughterless* in SL2 cells induces a myogenic phenotype. Cells were stained for DNA with Hoechst (4, 5, and 6) and anti-myosin heavy chain antibody (1, 2, and 3). Multinucleated syncytia expressing myosin fibrils are detected in transfected cultures, indicating myogenic conversion.

shown). When reverse transcriptase was not included in the RT-PCR, no product bands were observed (Fig. 3A, lanes 14–16), and DNA alone gave products consistent with the unspliced transcripts for *nautilus*, *daughterless*, and DMEF2. We also noted that the level of *daughterless* mRNA expression in Schneider cells appeared to be substantially lower than that of *nautilus* mRNA or the level of *daughterless* expression observed in embryos. In addition, two alternatively spliced forms of DMEF2 were detected in both embryos and in Schneider cells but each of the transcripts was expressed at a somewhat different level (Gunthorpe *et al.*, 1999; Taylor *et al.*, 1995). To confirm our observations concerning the relative levels of *nautilus* and *daughterless* mRNA in Schneider cells, we set up a more quantitative assay using 10-fold dilutions of total RNA and primers flanking introns in both *daughterless* and *nautilus* in quantitative RT-PCR. Using quantitative image analysis with the Fuji LAS1000 CCD camera and Cybergreen to

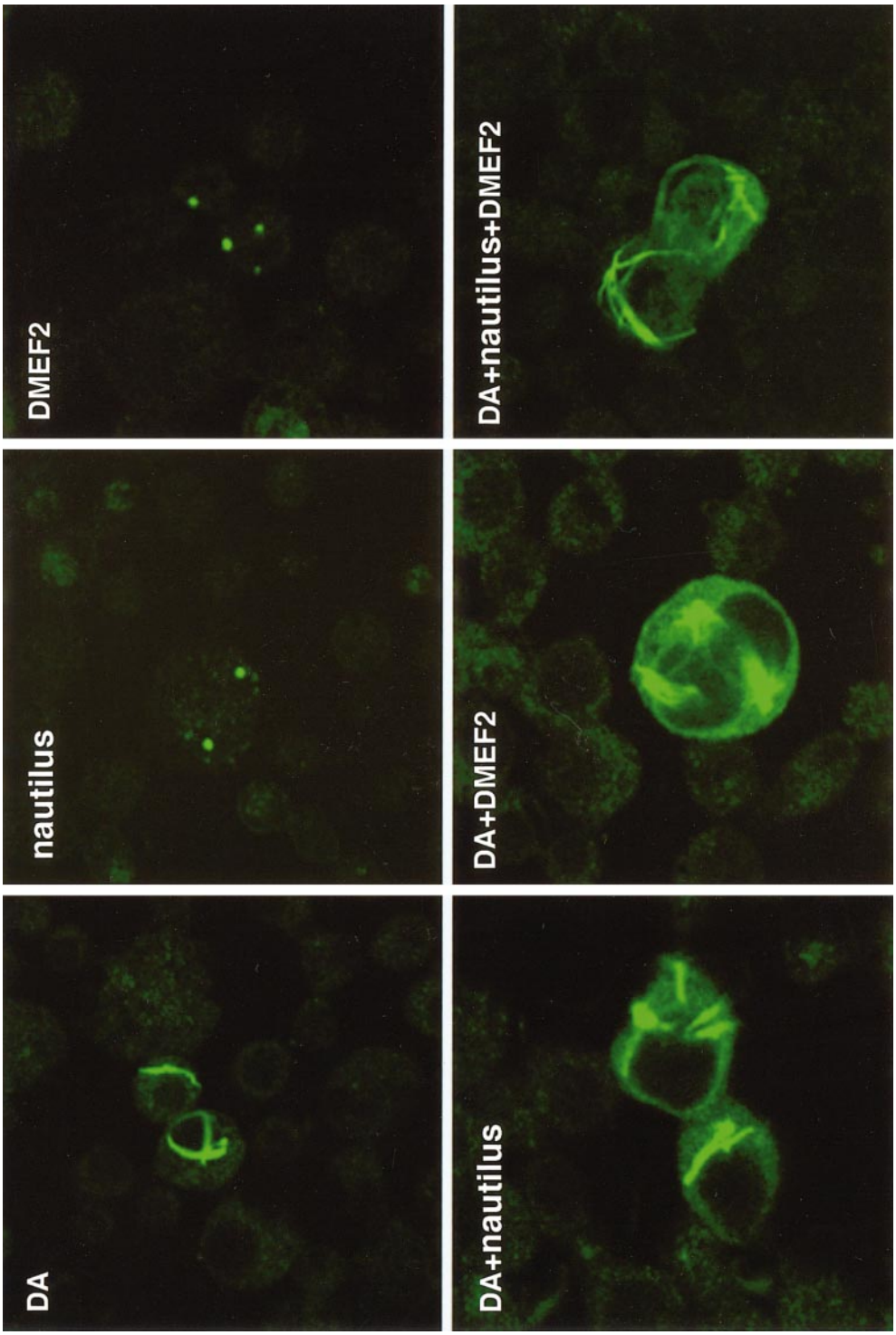

FIG. 2. Ectopic expression of either *nautilus* or DMEF2 does not induce fusion or myosin fibril formation but they can synergize with *daughterless* to increase myogenic conversion. The cells transfected with either *nautilus* or DMEF2 expression plasmids show low levels of myosin heavy chain expression in a punctate pattern (top middle and right) but no fusion or myosin fibrils are seen as in the cells expressing ectopic *daughterless* alone (top left). However, coexpression of *daughterless* with *nautilus* increases conversion 10-fold (Table 1) and this is further enhanced to 40-fold if *nautilus* is included with DMEF2. Myosin staining is never observed in normal-passaged or mock-transfected Schneider cells.

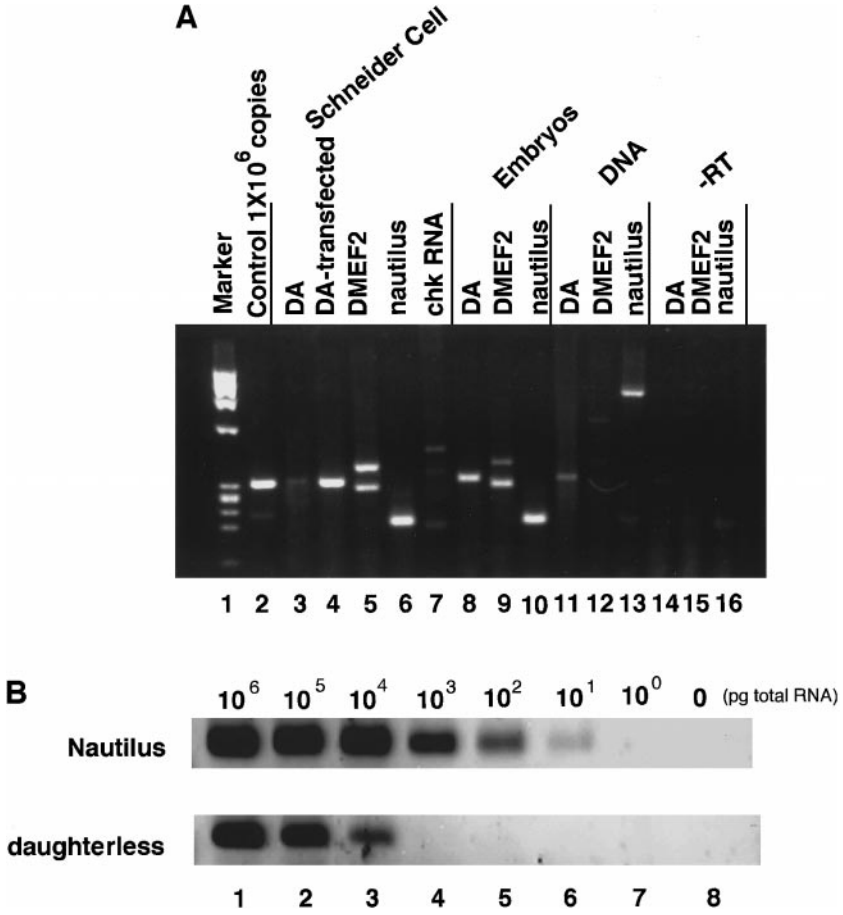

FIG. 3. SL2 cells express the mesodermally restricted mRNA transcripts for *nautilus* and DMEF2 as well as low levels of *daughterless* mRNA. (A) RT-PCR analysis of transcript levels in total RNA from SL2 cells with primers for the indicated genes: lane 1, *Hae*III ϕX DNA marker; lane 2, control RNA (10^6 copies per reaction); lanes 3–6, 1 μg total SL2 cell RNA with primers indicated above each lane; lane 7, 1 μg of total chicken RNA (chk) from myoblast cultures assayed with *nautilus* primers; lanes 8–10, 1 μg of total RNA from 0- to 16-h embryos with primers indicated above each lane; lanes 11–13, reactions with DNA only and indicated primers; lanes 14–16, no reverse transcriptase in the reaction with the indicated primers. Reactions carried out with DNA alone give products that are consistent with the unspliced mRNA transcripts. (B) *nautilus* mRNA transcripts are 100- to 1000-fold more abundant than *daughterless* transcripts; 10-fold dilutions of total SL2 RNA were analyzed by RT-PCR using primer pairs that span introns in *nautilus* and *daughterless* and gave similar-length PCR products of 320–350 bp.

detect the amplified bands, we estimated that the level of *daughterless* mRNA in Schneider cells is 10^2- to 10^3-fold lower than that of *nautilus* mRNA (Fig. 3B). In a second approach, in order to demonstrate that Schneider cells were expressing mRNAs that contained the full coding regions for *nautilus* and DMEF2, RT-PCR was performed using primers flanking the initiator methionine and terminator for the *nautilus* and DMEF2 coding regions with a T7 RNA polymerase binding site adjoined to the 5′ primer. DNA from these reactions was gel purified and subjected to *in vitro* transcription/translation analysis and immunoprecipitation to see if either *nautilus* or DMEF2 protein was correctly encoded by either amplified DNA. As shown in Fig. 4, full-size antibody-reactive *nautilus* and DMEF2 proteins were produced *in vitro* from both RT-PCR products. This result supported our assumption that Schneider cells express low levels of *nautilus* and DMEF2 protein. Based upon this observation and the very low levels of *daughterless* mRNA relative to *nautilus*, we concluded that the ectopic expression of *daughterless* activates the myogenic program in Schneider cells by raising the levels of *daughterless* protein sufficiently to promote the formation of the *nautilus*/*daughterless* heterodimer. *nautilus* activity is further enhanced by the presence of DEMF2, as mentioned above (Table 1).

In order to test this hypothesis, we decided to eliminate endogenous *nautilus* and DMEF2 gene function with RNA interference (Fire *et al.*, 1998). We had previously shown

TABLE 1

Synergy between *daughterless*, *nautilus*, and DMEF2 in the Myogenic Conversion of SL2 Cells

DA	Nau	DMEF2	Number of myosin-positive cells[a]
+			14
	+		0
		+	0
+	+		195
+		+	208
	+	+	0
+	+	+	545

Note. *Drosophila* SL2 cells cultured in HYQ-CCM3 medium were transfected with 1 μg of the indicated expression vectors. Total amount of transfected expression vehicle was normalized to 3 μg with the addition of PActPL expression vector DNA.

[a] The number of myosin-positive cells per 2×10^4 cells. The results represent the averages of three experiments.

that the injection of dsRNAs for *daughterless*, *twist*, *engrailed*, S59, and DMEF2 into *Drosophila* embryos gave results consistent with the known mutant phenotypes for these genes, while injection of *β-galactosidase* dsRNA had no phenotype. More importantly, injection of *nautilus* dsRNA, representing either the entire coding region or just the amino terminus, the bHLH region, or the carboxy terminus of the protein, severely affected muscle formation in the embryo and resulted in lethality (Misquitta and Paterson, 1999). Accordingly, we postulated that RNA-i would work when applied to Schneider cells.

Application of RNA-i to Schneider Cells

We assumed that dsRNA would behave like double-stranded DNA in transfections, so cellular delivery of dsRNA was performed by transfection with a lipophilic reagent rather than by the more complex injection procedure. Although the addition of dsRNA to mammalian cells induces the PKR-interferon pathway, resulting in apoptotic cell death (Williams, 1999; Zamanian-Daryoush *et al.*, 2000), this seemed unlikely to occur in Schneider cells given the embryo's response to injected dsRNA (Kennerdell and Carthew, 1998; Misquitta and Paterson, 1999). For the initial studies two test plasmids encoding cytoplasmic *β-galactosidase* and *nautilus*, a nuclear protein, were cotransfected along with dsRNA for one or the other of these genes. The first results demonstrated that transfected dsRNA, as predicted, did not kill insect cells and that expression of the cotransfected markers could be silenced by the corresponding dsRNA. As shown in Fig. 5A (1–3), cotransfected cells express both cytoplasmic *β-galactosidase* and nuclear *nautilus*. With the addition of dsRNA for *β-galactosidase* only nuclear *nautilus* staining is observed in the cotransfected cells (Fig. 5B4–6), whereas with the addition of dsRNA for *nautilus* only cytoplasmic *β-galactosidase* activity was detected (Fig. 5C7–9). Cotransfection of both dsRNAs for *nautilus* and *β-galactosidase* silenced expression of both genes (data not shown).

Protein Half-Life and the Effectiveness of RNA-i

An issue that occurred to us when considering the application of RNA-i in Schneider cells was the stability of the target protein in question. We speculated that stable proteins would be difficult to silence, whereas proteins that turned over more frequently would be amenable to silencing. To determine how protein stability affected specific gene silencing, cells were first transfected with an expression plasmid for either *β-galactosidase* or *nautilus*, then dsRNA for each protein was added to the cultures 24 h later and allowed to incubate a further 48 h prior to fixation.

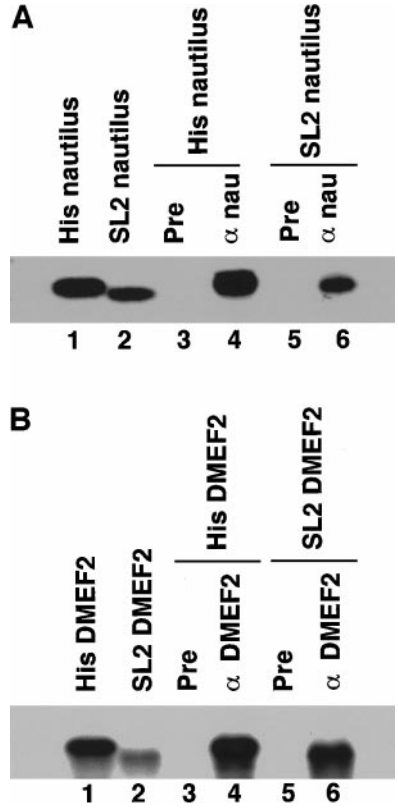

FIG. 4. The full-length *nautilus* and DMEF2 RT-PCR products encode the corresponding proteins. The complete *nautilus* and DMEF2 mRNAs were amplified by RT-PCR using 5′ primers with a T7 RNA polymerase binding site. The DNA products were then gel purified and transcribed/translated *in vitro* along with His-tagged *nautilus* and DMEF2 as controls in the pRSetA vector. (A) *nautilus* or (B) DMEF2; total product (lanes 1 and 2) was immunoprecipitated with preimmune (lanes 3 and 5), rabbit anti-*nautilus*, or anti-DMEF2 antibody (lanes 4 and 6).

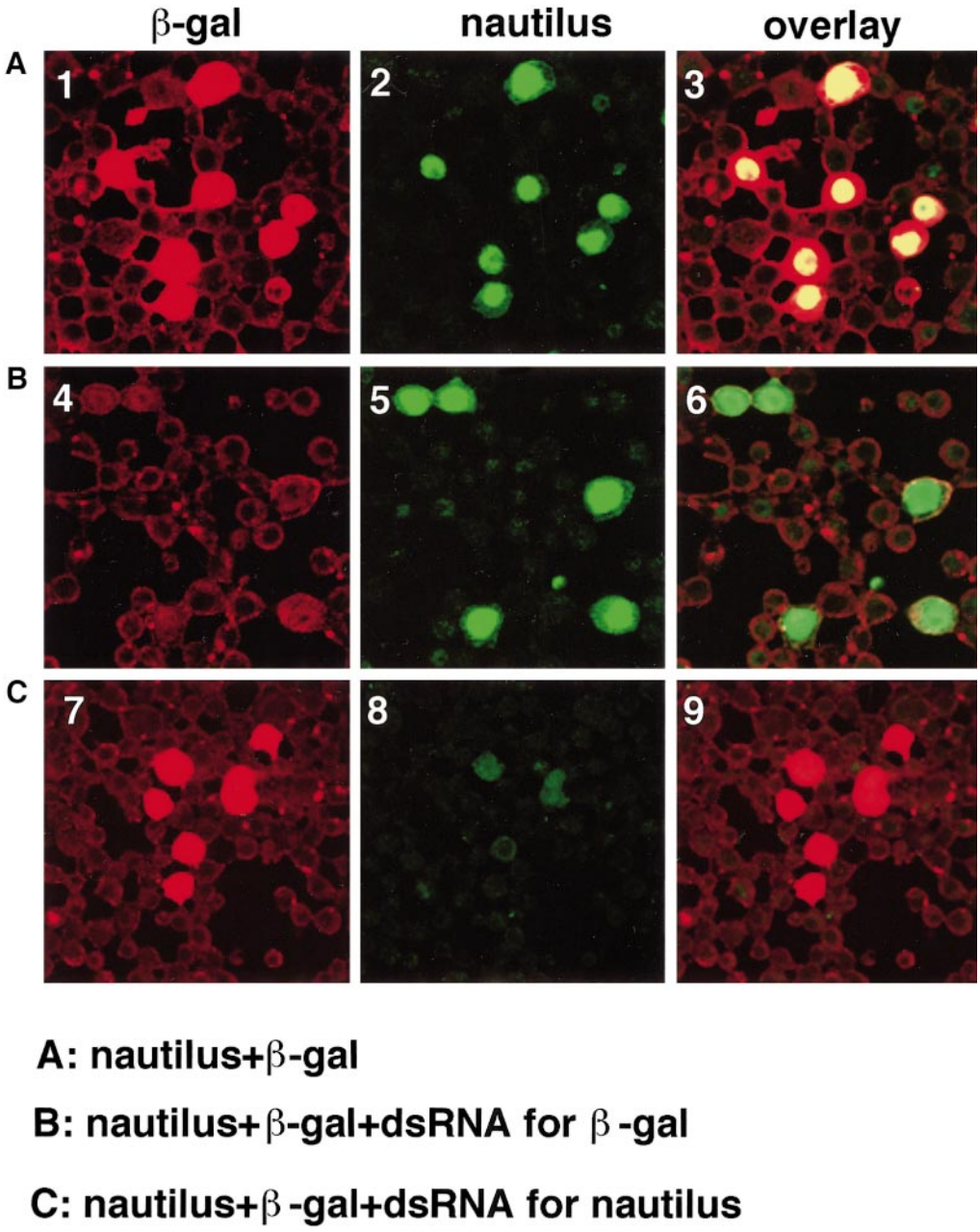

FIG. 5. Cotransfected dsRNA (RNA-i) can silence gene expression specifically in SL2 cells. SL2 cells were cotransfected with expression plasmids for *β-galactosidase* and *nautilus* alone (A1–3) or along with dsRNA for *β-galactosidase* (B4–6) or dsRNA for *nautilus* (C7–9). Expression was analyzed using monoclonal antibody against *β-galactosidase* (red) and rabbit polyclonal antibody against *nautilus* (green). Coexpression is shown as yellow. dsRNA specifically silences gene expression from the corresponding expression plasmid.

β-galactosidase is a very stable protein that is often used in conjunction with tissue-specific promoters to track cells that are expressing a gene of interest during development long after the endogenous gene has been silenced. This approach was used to mark the fate of cells expressing *nautilus* in the developing *Drosophila* embryo to demonstrate that each embryonic muscle fiber included at least one cell that expressed *nautilus* during development, even though the protein itself was no longer detectable in differentiated muscle (Paterson *et al.*, 1991). *nautilus*, like other members of the MyoD family of proteins, is assumed to be relatively unstable, with a half-life similar to that of the

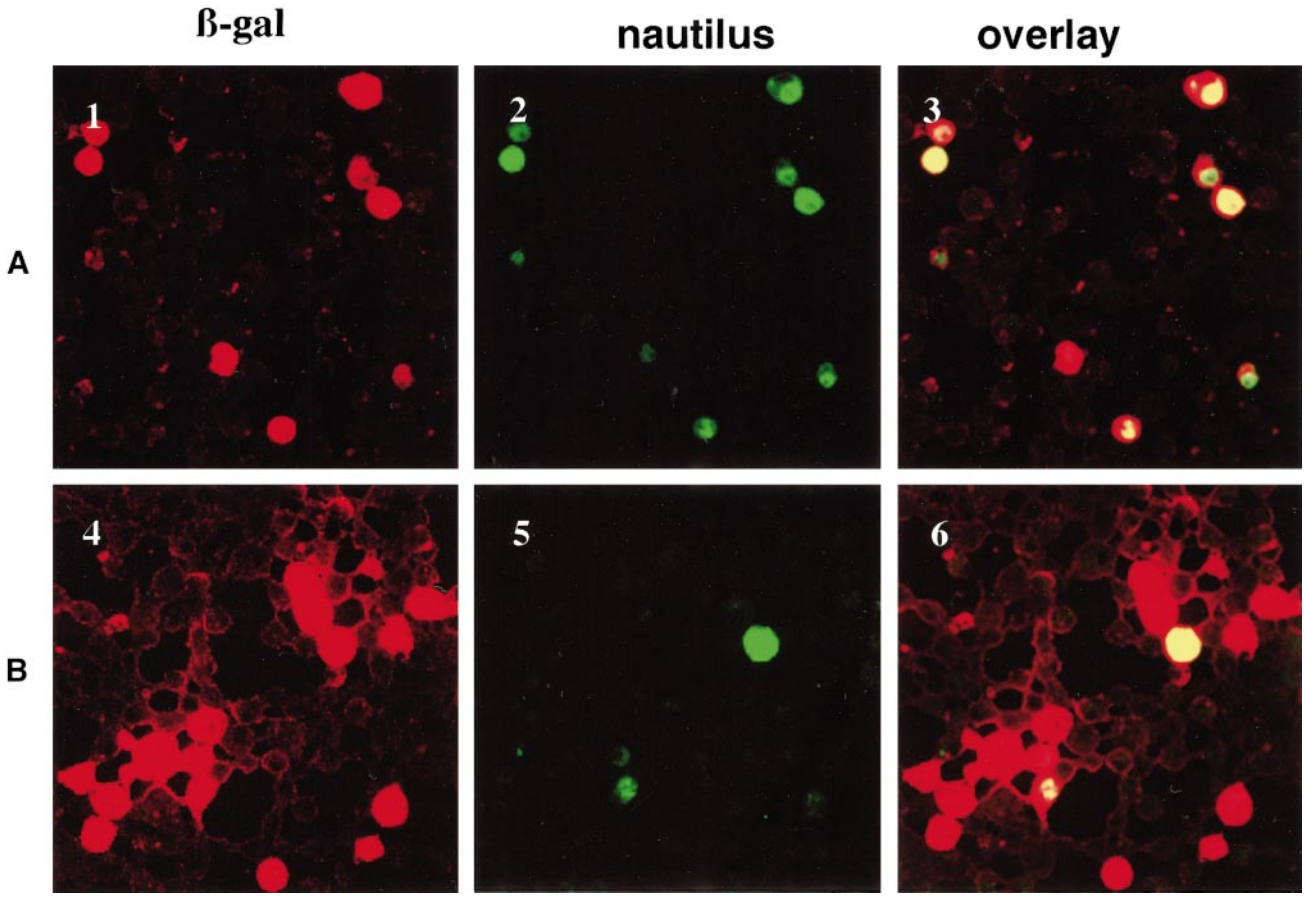

FIG. 6. The effectiveness of RNA-i depends upon the stability of the protein expressed from the target gene. Cells were cotransfected with plasmids expressing *β-galactosidase* and *nautilus*. 24 h later cells were either untreated (A) or treated (B) with dsRNA for *nautilus* and analyzed for expression of both proteins using antibodies as mentioned. Compared to the control cells in A, only 30% of the cells treated with *nautilus* dsRNA now express *nautilus* protein, whereas expression of *β-galactosidase* is unchanged (see Table 2).

vertebrate MyoD protein, around 30 min (Kitzmann *et al.*, 1999; Song *et al.*, 1998), although this has not been determined formally. However, similar serine–proline clusters that are phosphorylated in vertebrate MyoD to regulate the half-life of the protein are also noted in *Drosophila nautilus* (Michelson *et al.*, 1990; Paterson *et al.*, 1991). As predicted, levels of *β-galactosidase* expression were essentially unchanged, whereas *nautilus* expression was reduced by more than 70% in the cells treated with dsRNA, reflecting the intrinsic turnover of each protein (Fig. 6 and Table 2). In contrast, when dsRNAs for *nautilus* and *β-galactosidase* were cotransfected with the corresponding reporter plasmid no expression of either reporter was detected (see above), indicating that the RNA interference effect is immediate under these conditions. These results suggest that posttranscriptional gene silencing by dsRNA depends substantially upon the turnover time of the protein in question and this, in turn, will impact efforts to silence particular genes.

TABLE 2

Gene Silening by RNA-i Depends upon Protein Stability

dsRNA with plasmid DNA	ds RNA 24 h after plasmid DNA	% Cells expressing *β-gal* or *nautilus*
—	—	100 *β-gal*
β-gal	—	0 *β-gal*
—	*β-gal*	94 *β-gal*
—	—	100 *nau*
nau	—	0 *nau*
—	*nau*	31 *nau*

Note. Cells were transfected with expression plasmid for *β-gal* and *nautilus*. dsRNA was then added either with plasmid DNA or 24 h after plasmid transfection. Cells were scored as the number of *β-gal*-positive cells per 500 *nautilus*-positive cells, in the case of *β-gal* dsRNA addition, and visa versa in the case of nautilus ds RNA.

Silencing Endogenous Gene Expression in Schneider Cells with dsRNA

Having shown that the transfection of dsRNA into Schneider cells can silence ectopically expressed genes, we wanted to test the ability of RNA-i to silence the expression of selected endogenous proteins in Schneider cells. As test examples, we chose heat-shock factor (HSF), the activator of Hsp26; cytoplasmic actin, a relatively stable structural protein; and β-tubulin. HSF is a well-characterized transcription factor with a half-life of approximately 8 to 10 h (Fritsch and Wu, 1999). Cultures treated with HSF dsRNA had greatly reduced endogenous expression levels of HSF in 80–90% of the cells, as determined by HSF antibody staining (Fig. 7A). This reduction was also noted in the HSF protein level determined by the Western blot analysis of whole-cell extracts (Fig. 7B). As a consequence of the HSF silencing by dsRNA, activation of Hsp26 by HSF after heat shock was also greatly diminished, whereas tubulin expression levels in the treated cells were essentially unaffected (Fig. 7B). Similar inhibition of HSF function was observed when HSF dsRNA was used to block the heat-shock activation of an HSF reporter plasmid (data not shown). Very few cells cotransfected with dsRNA for cytoplasmic actin and a nuclear GFP marker gave any phenotype or change in cell number but where it was observed cells were typically very flattened with diminished actin staining compared to cells transfected with GFP alone (data not shown). Cells marked with nuclear GFP and treated with dsRNA for β-tubulin were reduced sixfold in number compared to cells treated with the GFP vector alone, suggesting that β-tubulin turns over with a shorter half-life than actin and that loss of β-tubulin is lethal (data not shown).

An Essential Role for nautilus and DMEF2 in the daughterless-Dependent Myogenic Conversion of Schneider Cells

Since Schneider cells appeared to express functional *nautilus* and DMEF2 mRNAs, we wanted to determine whether myogenic conversion by *daughterless* was dependent upon the endogenous activity of these genes. In order to test this idea we cotransfected dsRNA for *nautilus* and DMEF2 along with nuclear *β-galactosidase* to mark transfected cells. Twenty-four hours later the cells were again transfected with the *daughterless* expression plasmid and scored for the myogenic conversion of *β-galactosidase*-positive cells after an additional 48 h of incubation. In cells pretreated with either *nautilus* or DEMF2 dsRNA, ectopic expression of *daughterless* was not able to trigger myogenic conversion (Fig. 8, center and right). However, cells not treated with either dsRNA contained the typical myosin fibrils and were clearly converted to a myogenic phenotype (Fig. 8, left). Therefore, activation of the myogenic program through *daughterless* requires both functional *nautilus* and functional DEMF2, similar to the role for these proteins in *Drosophila* myogenesis, as determined by mutational analysis and RNA interference (Lilly *et al.*, 1995; Misquitta and Paterson, 1999).

DISCUSSION

Transgenic and gene-targeting studies in the mouse have clearly shown that the process of myogenesis in the vertebrates is dependent upon the activity of the MyoD family of gene regulatory factors, MyoD, myf-5, myogenin, and MRF4/herculin (see previously mentioned reviews). In all the invertebrate systems studied to date there is but a single MyoD-related gene. In *C. elegans* the *hlh-1* gene is essential for complete myogenesis and viability (Chen *et al.*, 1994). However, in *Drosophila* there is disagreement on the role of *nautilus* as an essential myogenic factor. Transheterozygous deficiencies that in combination are reported to remove *nautilus* did not affect survival nor was somatic muscle formation substantially impacted except in muscles 3 and 19 (Keller *et al.*, 1998). This was interpreted to suggest that the *nautilus* gene product is not essential for viability and that *nautilus* functions only in the formation of a small subset of embryonic muscles. However, several results argue against this interpretation. First, injection of *nautilus* dsRNA into embryos (RNA-i) as well as expression of *nautilus* antisense RNA in the mesoderm using the Gal4/UAS system both resulted in a severe loss or absence of muscle in the embryo (Misquitta and Paterson, 1999); second, ricin toxin ablation of *nautilus*-expressing cells completely disrupts the muscle pattern, not just muscles 3 and 19 (Misquitta and Paterson, 1999); third, *nautilus*-expressing cells are incorporated into essentially every somatic muscle in the embryo, as determined by *β-galactosidase* expression from the *nautilus* promoter driving LacZ, and *nautilus* antibody staining is seen in several newly formed somatic muscles other than muscles 3 and 19, notably muscles 12, 15, 16, 17, 26, and 27 (Paterson *et al.*, 1991). The reason for the discrepancy between the genetic study and our results is not clear. However, in the absence of a true *nautilus* null that eliminates both maternal and zygotic expression the controversy will not be resolved. The different results may reflect the presence of an unknown related gene. However, this possibility is not considered likely since injection of dsRNA representing the full *nautilus* coding region, the amino terminus, the bHLH domain, or the carboxy terminus of the protein all gave severe loss-of-muscle phenotypes. Furthermore, searches in the *Drosophila* genome data base have also not turned up any closely related genes, either at the nucleotide level or at the amino acid level, that show sufficient homology to support dsRNA gene silencing. A recently published report on a genome-wide survey of basic helix-loop-helix factors in *Drosophila* supports this conclusion (Moore *et al.*, 2000).

A hallmark for the activity of the vertebrate myogenic regulatory proteins is the ability of each factor to convert nonmuscle cells to a myogenic fate (Choi *et al.*, 1990; Davis

et al., 1987; Weintraub *et al.,* 1989). The MyoD-related proteins from invertebrates, including star fish, ascidians, *C. elegans,* and *Drosophila,* share this property. However, *Drosophila nautilus* cannot heterodimerize efficiently with the vertebrate E proteins to form an active heterodimer (Shirakata *et al.,* 1993), so myogenic conversion does not occur in the classical mouse fibroblast model, even though cell nuclei stain positively with *nautilus* antibody. *Drosophila daughterless,* the E-protein homologue, must be supplied ectopically with *nautilus* to effect the myogenic conversion of mouse fibroblasts, presumably through the formation of the *nautilus/daughterless* heterodimer (Zhang *et al.,* 1999). Likewise, misexpression of *nautilus* in the *Drosophila* embryo, using the Gal4/UAS system (Brand and Perrimon, 1993), has clearly demonstrated that *nautilus* can transform cardiac cells to somatic muscle as well as alter the pattern and identity of some somatic muscle fibers (Keller *et al.,* 1997). Most importantly, the majority of the MyoD-related factors that have been characterized in both the vertebrates and the invertebrates are capable of activating the myogenic program in a variety of heterologous cell backgrounds and most, with the exception of *C. elegans hlh-1,* function as heterodimers with E-related proteins. The results reported here demonstrate that *daughterless* requires *nautilus* to activate the myogenic program in Schneider cells, presumably as a heterodimer, and we argue that this is likely to be the case during *Drosophila* development. No other group of muscle regulatory factors shares this property, making the MyoD family of proteins unique positive regulators of the myogenic pathway during development. If the genetic analysis of *nautilus* function is correct (Keller *et al.,* 1998), this would be the only example of normal muscle development in the complete absence of a MyoD-related protein. We favor the idea that *nautilus* marks the subset of muscle precursor cells, or founders (Bate, 1990; Baylies *et al.,* 1998), that establish the muscle pattern in each hemisegment and that these cells recruit fusion-competent mesodermal cells to complete muscle formation. Final activation of the myogenic program, we suggest, requires *nautilus* expression in every muscle and our *in vivo* and *in vitro* data support this model. Two recent reports describe the characterization of the immunoglobulin-related genes, *duf* and *sns,* that are expressed in founder and fusion-competent myoblasts, respectively, during *Drosophila* myogenesis (Bour *et al.,* 2000; Ruiz-Gomez *et al.,* 2000) and both genes are essential for myoblast fusion. It is not clear if *nautilus* expression is restricted to *duf*-positive myoblasts but our results would predict this to be the case, based upon the lack of muscle development in embryos ablated for the *nautilus*-expressing cells (Misquitta and Paterson, 1999). We do not know if *duf* or *sns* are also expressed in myogenically converted Schneider cells but this will be examined further.

The failure to form well-organized muscle structure in the converted Schneider cells may be a property of the SL2 cell line itself since it is 60 to 80% tetraploid (Echalier, 1997). A similar lack of sarcomeric structure is also observed in mouse 10T1/2 fibroblasts converted to muscle with the transient expression of MyoD. One typically sees clusters of nuclei in a myosin-positive syncytium but no organized sarcomeres with defined Z lines. The observation that most of the Schneider cell syncytia expressing myosin contain two nuclei suggests that the daughter cells from a recently converted cell may have fused to form a binucleate. The syncytia containing three or more nuclei represent a much smaller percentage of the converted cell population. However, cultured *Drosophila* embryonic muscle fibers typically contain four to six nuclei per myotube so this reduction in nuclei compared to vertebrate muscle fibers may be a general property of embryonic *Drosophila* muscle formation (Paterson *et al.,* 1991; Storti *et al.,* 1978). The possible absence of *duf* or *sns* expression may also limit the degree of cell fusion observed. More importantly, these syncytia with two or more nuclei never show labeling with BrdU so they arise by cell fusion and not endoreplication (unpublished observations).

The *daughterless* activation of the myogenic program in Schneider cells initially hinted that there might be a novel pathway in *Drosophila* myogenesis independent of *nautilus* gene activity. *daughterless* had been previously implicated in myogenesis since homozygous null embryos show severe muscle defects (Caudy *et al.,* 1988). However, RT-PCR analysis revealed that full-length, spliced *nautilus* and DMEF2 mRNAs are present in Schneider cells and that both are capable of encoding *nautilus* and DMEF2 proteins, even though the endogenous proteins themselves cannot be detected by Western analysis or antibody *in situ* reactions.

daughterless, like the vertebrate E protein E12, has an acidic domain amino-terminal to the basic region that inhibits homodimer formation (Shirakata and Paterson, 1995), so it was considered unlikely that *daughterless* could act as a homodimer in the Schneider cell myogenic conversion. Quantitative RT-PCR analysis further revealed that *daughterless* expression levels were substantially lower (100- to 1000-fold) compared to *nautilus* so the cellular concentration of *daughterless* would likely be a limiting factor in the formation of the active *nautilus/daughterless* heterodimer. *daughterless/nautilus* heterodimers readily form *in vitro* and bind efficiently to an E-box consensus in gel-shift assays, whereas either monomer alone binds DNA poorly (Shirakata and Paterson, 1995; Zhang *et al.,* 1999). Our results support the conclusion that ectopic *daughterless* expression activates myogenesis in Schneider cells by raising the endogenous levels of the active *nautilus/daughterless* heterodimer. Consistent with this interpretation, ectopic expression of either *nautilus* alone or in combination with DMEF2 in Schneider cells did not activate the myogenic conversion observed with *daughterless.*

twist has been shown to play an essential role in mesodermal differentiation and myogenesis (Baylies and Bate, 1996; Baylies *et al.,* 1997); therefore, we wanted to determine if Schneider cells expressed *twist* since these cells have myogenic potential and are of mesodermal origin, based upon marker expression. However, we were unable to detect spliced *twist* mRNA in our RT-PCRs so myogenic

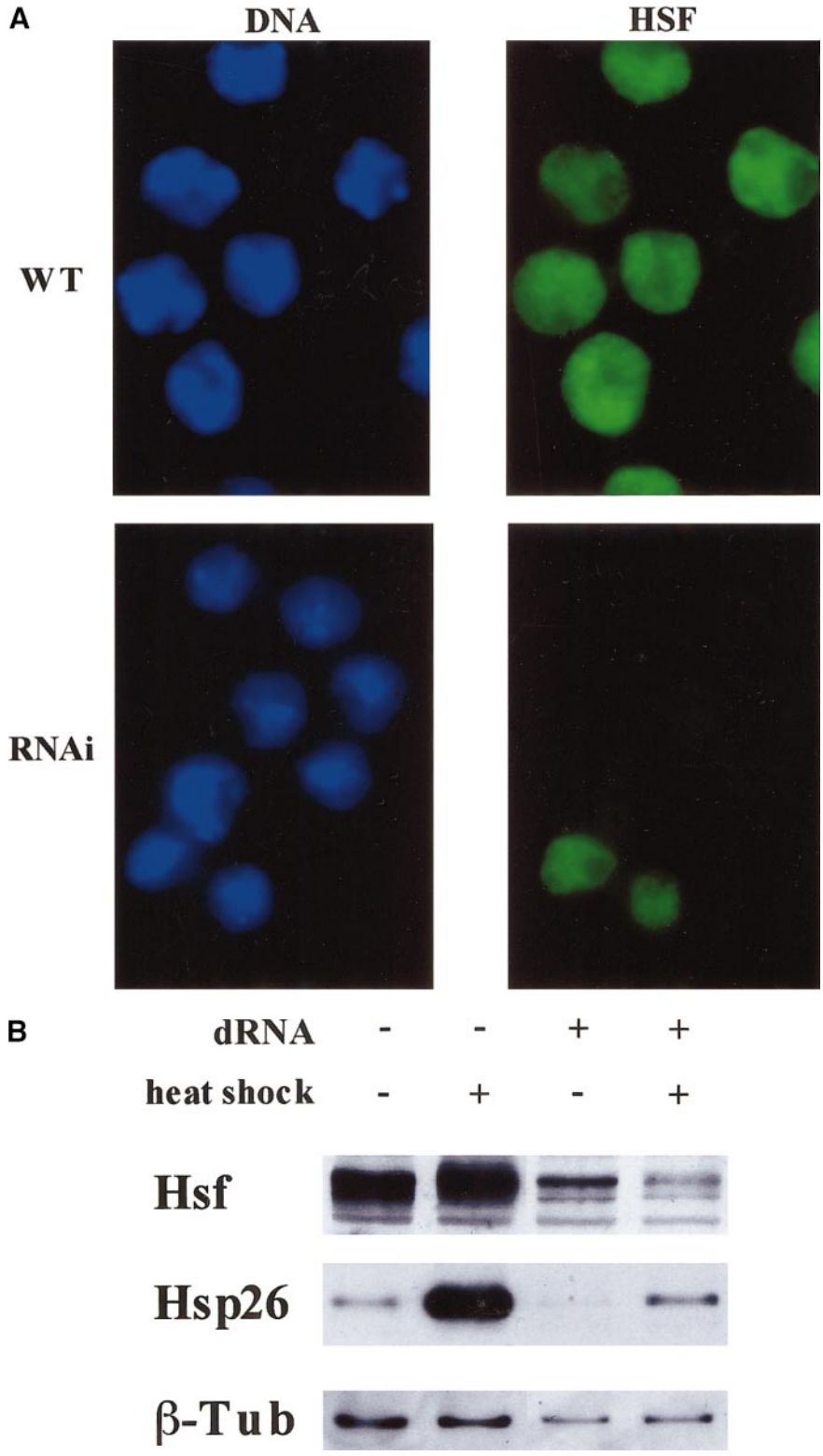

FIG. 7. Depletion of endogenous heat-shock factor (HSF) in SL2 cells by RNA-i and the subsequent inhibition of Hsp26 induction by heat shock. Cells were transfected with (+) or without (−) double-stranded RNA against the first 1800 bp of the HSF coding region. (A) Two days after transfection cells were analyzed for HSF expression levels by *in situ* staining with HSF antibody in wild-type (WT) cells and in cells treated with dsRNA for HSF (RNAi). (B) At the same time whole-cell extracts were analyzed for HSF, Hsp26, and β-tubulin expression levels by Western blot analysis before and after heat shock in the presence and absence of dsRNA for HSF.

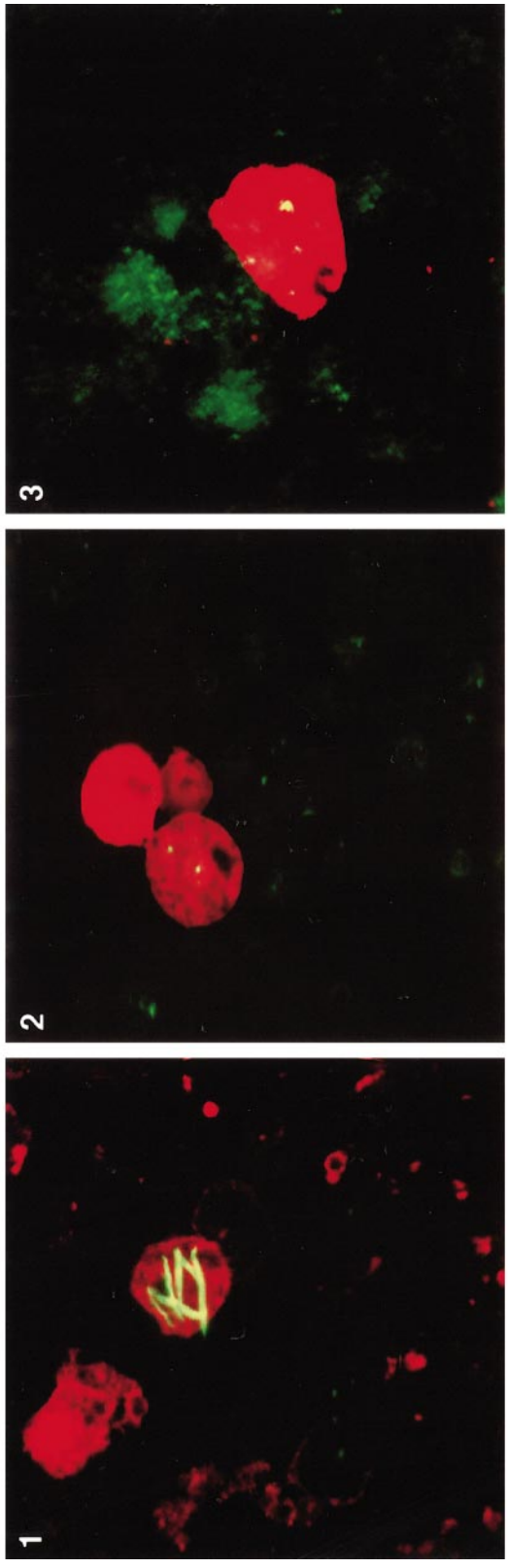

FIG. 8. Myogenic conversion of SL2 cells depends upon endogenous *nautilus* and DMEF2 protein. SL2 cells were transfected with a *β-galactosidase* expression plasmid plus or minus dsRNA for *nautilus* or DMEF2. 24 h later the same cells were again transfected with the *daughterless* expression plasmid. Cells that received no *nautilus* or DMEF2 dsRNA express myosin heavy chain that assembles into fibers (left), whereas the cells that received either *nautilus* (middle) or DMEF2 (right) dsRNA show only punctate myosin staining.

conversion of Schneider cells does not appear to require active *twist*. *twist* will heterodimerize with *daughterless* so heterodimer formation would be postulated to repress myogenesis triggered by ectopic *daughterless* expression. This is in fact what was observed in our initial experiments. Coexpression of both *twist* and *daughterless* in Schneider cells blocked the myogenic conversion by *daughterless* (data not shown). A similar result has also been noted with vertebrate *twist* and MyoD (Spicer *et al.*, 1996). Furthermore, cotransfection of dsRNA for *twist* along with the *daughterless* expression plasmid did not inhibit myogenic conversion by *daughterless*. This last result is consistent with the RT-PCR studies that indicated that no *twist* mRNA is expressed in Schneider cells and that active twist is not required for conversion. However, this result does not rule out a role for *twist* prior to the onset of *nautilus* function in the activation of the muscle program in Schneider cells. In the embryo, *twist* expression is lost from cells as they differentiate, consistent with the idea that *twist* is not essential for activation of the myogenic program, per se. Schneider cells likely represent a transition state in the process of myogenesis since the cells express no *twist* and very low levels of *nautilus*. We have not checked to see if other potential muscle markers are expressed in SL2 cells such as S59, *Kruppel*, or *apterous*, but this may be unlikely since these genes are expressed in an even more restricted subset of muscle precursor cells (Bourgouin *et al.*, 1992; Dohrmann *et al.*, 1990; Ruiz-Gomez *et al.*, 1997).

In order to demonstrate that *nautilus* and DMEF2 were acting as essential cofactors with *daughterless* in the myogenic conversion of Schneider cells, we had to show that conversion was dependent upon both *nautilus* and DMEF2 gene function. We decided to test whether RNA interference could be applied to cultured insect cells since it was so effective in knocking out both *nautilus* and DMEF2 gene function, disrupting muscle formation in the *Drosophila* embryo (Misquitta and Paterson, 1999). The transfection of dsRNA into Schneider cells with lipophilic agents proved to be a very effective way to silence transfected reporters as well as endogenous gene function in the examples we tested. Most importantly, if dsRNA for either *nautilus* or DMEF2 was transfected into Schneider cells 24 h prior to the ectopic expression of *daughterless*, no *daughterless*-dependent myogenic conversion occurred. Thus conversion is dependent upon the presence of both *nautilus* and DMEF2 and presumably involves the formation of the *daughterless/nautilus* heterodimer, all of which have been shown to be essential genes in *Drosophila* myogenesis, based upon well-characterized mutations and the application of RNA-i (Caudy *et al.*, 1988; Cronmiller *et al.*, 1988; Lilly *et al.*, 1995; Misquitta and Paterson, 1999). This result is similar to the *daughterless*-dependent conversion of mouse fibroblasts by *nautilus* in that the presence of both proteins is necessary to trigger conversion, presumably through the formation of the heterodimer (Zhang *et al.*, 1999). This conversion in mammalian cells probably requires endogenous MEF2 but this has not been formally established.

In support of our findings, two recent reports have also demonstrated the utility of RNA interference in gene silencing in Schneider cells, one describing loss-of-function phenotypes for cyclins A and E (Hammond *et al.*, 2000) and the second dissecting signal transduction pathways (Clemens *et al.*, 2000). In the latter case, Schneider cells were simply shaken vigorously with high concentrations of dsRNA for 30 min in serum-free medium and this led to effective silencing in 95–99% of the cells. This is similar to the silencing efficiency we observed with HSF dsRNA and the loss of HSF protein expression measured by antibody staining of the cells (Fig. 7). Loss of HSF function by RNA-i in Schneider cells is not lethal and this agrees with the previously published mutant phenotype which has shown that HSF is dispensable for general cell growth and viability in *Drosophila* (Jedlicka *et al.*, 1997). It has been previously reported that plasmid uptake will reach more than 95% while no more than 30% of the cells will express the transgene at 24 h (Tseng *et al.*, 1997). Silencing may be relatively more efficient compared to transgene expression since so few molecules of dsRNA are required to effect silencing (Fire *et al.*, 1998).

The tissue origins of the Schneider cell lines have been difficult if not impossible to establish since every line was derived from dissociated cells representing a large number of embryos of different ages. Our data suggest that the SL2 line is mesodermal in nature since it expresses the spliced mRNA transcripts for two well-characterized mesodermal markers, *nautilus* and DMEF2. Furthermore, these transcripts appear to be functional since they can encode the corresponding proteins *in vitro*. However, based upon this result alone, we cannot exclude the possibility that translational control may keep certain endogenous mRNAs silent. However, the fact that dsRNA for *nautilus* and DMEF2 can block *daughterless* conversion of Schneider cells argues that the proteins are in fact expressed at very low levels.

It is interesting to note that insect cells do not appear to have a lethal response to dsRNA in that they are not triggered to undergo apoptosis like mammalian cells (Williams, 1999). The dsRNA-activated kinase, PKR, found in mammalian cells that phosphorylates elongation factor and IκB to block protein synthesis and activate the NFκB/apoptosis pathway, respectively, is either absent or does not play the same role in insect cells (Williams, 1999). In confirmation of this observation, we have cotransfected Schneider cells with expression plasmids for mouse PKR and *β-galactosidase* along with dsRNA for *β-galactosidase* and in this instance the cells die (Wei, Williams, and Paterson, unpublished observations). This killing may involve the IκB pathway since *Drosophila* IκB does play a role in the insect immune response (Bernal and Kimbrell, 2000; Packman *et al.*, 1997).

We have used the same RNA-i protocol in mammalian cells but in every instance the cells do not survive. Even

cells from the PKR (−/−) mouse (Zamanian-Daryoush *et al.*, 2000) were susceptible to killing with dsRNA (Wei, Williams, and Paterson, unpublished observations). The reason for this killing is not clear but may be due to the presence of additional PKR-related kinases (Williams, 1999). Inclusion of the PKR inhibitor protein, P58, reduced cell killing somewhat in mouse 10T1/2 cells treated with dsRNA but we were unable to obtain specific gene silencing under these circumstances (Lee *et al.*, 1994). Gene silencing by RNA-i has been reported in zebrafish (Wargelius *et al.*, 1999) and more recently in mouse embryos (Wianny and Zernicka-Goetz, 2000). In both instances the dsRNA was injected into the developing embryo. Our recent experiments using dsRNA on ES cells suggest that the PKR system is active in embryonal cells so developmental timing is apparently not a factor explaining the successful application of RNA-i in vertebrate embryos. However, the injection of dsRNA into zebrafish embryos has been recently reported to have nonspecific effects so the utility of RNA-i in vertebrate systems is not that clear at the present time (Oates *et al.*, 2000).

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